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PHARMACOLOGICAL STUDIES ON CLOSTRIDIAL NEUROTOXINS

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Studies have been done that involve synthetic as well as naturally occurring clostridial neurotoxin antagonists. Ammonium chloride (1-8 mM) and methylamine hydrochloride (1-16 mM) produced concentration-dependent antagonism of the onset of neuromuscular blockade caused by botulinum toxin types A, B and C (all at 1 x 10^{-11} M) and by tetanus toxin (3 x 10^{-10} M). Neither drug antagonized the onset of paralysis caused by β-bungarotoxin (1 x 10^{-7} M) or by taipoxin (1 x 10^{-8} M). At concentrations that produced antagonism of clostridial neurotoxins, ammonium chloride and methylamine hydrochloride (8-10 mM) did not inactivate toxin molecules, nor did they produce irreversible changes in tissue function. When studied under conditions that impose partial synchrony on the mechanism of clostridial neurotoxin action, ammonium chloride and methylamine hydrochloride did not inhibit ligand binding and did not reverse neuromuscular blockade. The drugs acted solely to antagonize internalization of toxins by cholinergic nerve endings. As a result of inhibiting the process of internalization, the drugs trapped the toxins at an antitoxin sensitive site.

Tetanus toxin, fragment B and fragment C were assayed for toxicity on the mouse phrenic nerve-hemidiaphragm preparation. The native toxin was a potent blocker of neuromuscular transmission; fragment B possessed little toxicity and fragment C was atoxic. Pretreatment of tissues with fragment C antagonized the neuromuscular blocking properties of tetanus toxin, but not those of type A botulinum toxin or β-bungarotoxin. Fragment C exerted its effect by competing with unbound toxin for receptor sites on the nerve membrane. The fragment did not: i.) displace bound toxin, ii.) inhibit internalization of toxin, or iii.) inhibit intracellular expression of toxicity. In assays on intact cells, under conditions in which toxin binding was not dissociable, fragment C binding to phrenic nerves had an apparent K_D of ~1.4 x 10^{-7} M. Homogenates of mouse cerebral cortex adsorbed tetanus toxin, and these homogenates competed with phrenic nerves for unbound toxin. Homogenized cortex did not displace or promote desorption of toxin already bound to phrenic nerves. Homogenates of eel and torpedo electric organ were not very effective in adsorbing toxin.

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5. Progress Report

A. Synthetic antagonists of toxins.

Botulinum toxin and tetanus toxin are potent pharmacological substances that block the release of acetylcholine at the cholinergic neuromuscular junction (Burgen et al., 1949; Brooks, 1956; Habermann et al., 1980). No drug has been identified that reverses paralysis caused by these toxins, but several procedures have been shown to delay onset of paralysis. For example, lowering temperature, decreasing the rate of nerve stimulation, or modifying the ambient concentration of calcium can slow the development of toxin-induced neuromuscular blockade (Burgen et al., 1949; Thesleff, 1960; Hughes and Whaler, 1962; Habermann et al., 1980; Schmitt et al., 1981). All of these procedures exert their effects wholly or in part by altering the probability of transmitter release (DasGupta and Sugiyama, 1977; Simpson, 1981).

The purpose of the present report is to introduce a new class of drugs that can antagonize the onset of paralysis caused by clostridial neurotoxins. These drugs include ammonium chloride and a series of short chain alkylamines, for which methylamine hydrochloride is a representative member. These drugs do not act by modifying the probability of transmitter release from nerve endings; instead, they modify the probability of toxin entry into nerve endings.

The proposed mechanism of clostridial neurotoxin action, using botulinum toxin (BT) as a prototype, can be written as follows (Simpson, 1981):

1. $BT_o + R_1 \rightarrow BT_o \cdot R_1$
2. $BT_o \cdot R_1 \rightarrow BT_i + R_1$
3. $BT_i + R_2 \rightarrow BT_i \cdot R_2$

According to this scheme, botulinum toxin acts extracellularly (BT_o) to bind to a cell surface receptor (R_1). This complex is internalized, after which intracellular toxin (BT_i) separates from its membrane receptor. The toxin then binds to a second receptor (substrate?; R_2), and the result of this interaction is blockade of transmitter release.

Two classes of drugs have been found that antagonize the first reaction. Certain gangliosides antagonize the binding of botulinum toxin (Simpson and Rapport, 1971; Kitamura et al., 1980) and tetanus toxin (Van Heyningen and Miller, 1961) to nerve membranes. Gangliosides may be toxin receptors, and thus exogenous gangliosides compete with membrane gangliosides for the binding moiety on clostridial neurotoxins. In addition, polypeptides cleaved from native toxins antagonize the binding of clostridial neurotoxins (Kozaki, 1979; Morris et al., 1980). These polypeptides are believed to contain the fragments that recognize membrane receptors, so the polypeptides compete with toxins for tissue binding sites.

The present study describes a class of drugs that antagonize the second reaction. There are a number of pharmacologically active substances that must be internalized by target cells to exert their effects (Neville and Chang, 1978). In addition, there are drugs that are known to delay the onset of effect of these internalized substances. The drugs that

have been most carefully studied are chloroquine, ammonium chloride and methylamine hydrochloride (DeDuve et al., 1974; Goldstein et al., 1979; Pasten and Willingham, 1981). In a recent study, chloroquine was shown to delay the onset of neuromuscular blockade caused by botulinum toxin (Simpson, 1982). However, there are two limitations associated with the use of this drug. Chloroquine is itself a neuromuscular blocking agent, and this complicates its use as a botulinum toxin antagonist. Furthermore, chloroquine antagonizes only botulinum toxin; at usable concentrations it is not an antagonist of tetanus toxin. The present study was done to determine whether ammonium chloride and methylamine hydrochloride could antagonize botulinum toxin without themselves causing neuromuscular blockade. Beyond this, it was of interest to determine whether the drugs could antagonize tetanus toxin.

It has been found that both ammonium chloride and methylamine hydrochloride produce concentration-dependent antagonism of the onset of paralysis caused by botulinum toxin and tetanus toxin, but they do not antagonize other presynaptic toxins such as β-bungarotoxin or taipoxin. The concentrations of ammonium chloride and methylamine hydrochloride that antagonize clostridial neurotoxins are equivalent to those that produce antagonism of other protein toxins (e.g., Kim and Groman, 1965; Ivins et al., 1975), yet they are lower than those that produce neuromuscular blockade.

Ammonium chloride and methylamine hydrochloride do not act by inhibiting the binding of clostridial neurotoxins to

membrane receptors. When studied under conditions that permit toxin binding but which delay events after binding, neither ammonium chloride nor methylamine hydrochloride antagonized onset of neuromuscular blockade. Similarly, when studied under conditions in which binding and translocation had already gone to completion, the drugs once again lacked antagonistic activity. Ammonium chloride and methylamine hydrochloride exerted their protective effects only when they were present during the process of translocation.

Experiments with botulinum antitoxin support the concept that ammonium chloride and methylamine hydrochloride affect the process of internalization. When control tissues were exposed to botulinum toxin at a physiological temperature, the toxin disappeared from the neutralizing effects of antitoxin within 30 to 40 minutes. However, when tissues were treated with ammonium chloride or methylamine hydrochloride and then exposed to botulinum toxin, the toxin did not completely disappear from accessibility to antitoxin for 80 to 90 minutes. The most plausible explanation for these data is that clostridial neurotoxins must be internalized to exert their neuroparalytic effects, and ammonium chloride and methylamine hydrochloride act to inhibit the process of translocation.

There are two aspects of the data that deserve comment. The fact that ammonium chloride and methylamine hydrochloride antagonize botulinum toxin and tetanus toxin means they are unique drugs. They are among the first substances to be described that antagonize more than one class of presynaptic

toxin, and which do so by a mechanism other than altering the probability of transmitter release. In a somewhat different vein, there are many cell types that internalize ligands by the process of receptor mediated endocytosis, and whose mechanisms of endocytosis are vulnerable to the pharmacological actions of ammonium chloride and methylamine hydrochloride (Pastan and Willingham, 1981). The present study is the first to provide evidence that cholinergic nerve endings have a mechanism for endocytosis that is vulnerable to ammonium chloride and methylamine hydrochloride.

B. The binding fragment of tetanus toxin versus native tetanus toxin.

Tetanus toxin is an unusually potent substance that acts presynaptically to block neurotransmitter release (Bizzini, 1979; Wellhoner, 1982). In the central nervous system, the toxin acts at various sites to prevent release of gama-aminobutyric acid, glycine, norepinephrine and acetylcholine (Osborne and Bradford, 1973; Bigalke et al., 1981). In the peripheral nervous system, the toxin acts at postganglionic parasympathetic sites and at the neuromuscular junction to prevent release of acetylcholine (Ambache et al., 1948; Bigalke and Habermann, 1980; see Habermann et al., 1980, for references on neuromuscular transmission).

The precise mechanism by which the toxin inhibits transmitter release has not been fully determined. However, studies on the neuromuscular junction suggest that toxin-induced blockade involves a sequence of three reactions

(Schmitt et al., 1981). There is an extracellular binding step, an internalization step, and an intracellular lytic step.

The three reactions that account for the pharmacological effects of tetanus toxin can be related to three functional domains within the toxin molecule. Tetanus toxin is a 150,000 dalton protein that is composed of two polypeptide chains (heavy chain 100,000 daltons; light chain 50,000 daltons) that are linked by a disulfide bond (DasGupta and Sugiyama, 1977; Bizzini, 1979; Wellhoner, 1982; Robinson and Nash, 1982). The heavy chain mediates both receptor binding and internalization. More precisely, the 50,000 dalton carboxy-terminus of the heavy chain (fragment C) binds with high affinity to receptors in nerve tissue (Morris et al., 1980; Goldberg et al., 1981), and the 50,000 dalton amino-terminus of the heavy chain forms channels in membranes (Boquet and Duflot, 1982). The light chain acts intracellularly to block transmitter release, but the nature of the lytic step is unknown.

Studies on the binding of tetanus toxin and fragment C to nerve tissue have been done mainly on broken cell preparations from the central nervous system (Morris et al., 1980; Goldberg et al., 1981; Roger and Snyder, 1981). These studies have shown that native toxin and fragment C compete for the same receptor, and thus fragment C can occlude binding of the parent molecule. The purpose of the present report is to extend these findings to an intact cell preparation. Data

are presented which show that fragment C binds to the isolated phrenic nerve-hemidiaphragm preparation, and in doing so it antagonizes the neuromuscular blocking actions of native tetanus toxin.

In accordance with earlier findings (see Habermann et al., 1980, for brief review), tetanus toxin was shown to block neuromuscular transmission. Fragment C was devoid of toxicity, but fragment B was weakly toxic. Helting et al. (1978), who used an *in vivo* assay, have also presented evidence that fragment B is weakly toxic. These findings suggest that fragment B should be purified to homogeneity and then tested for activity on isolated neuromuscular preparations. Such work would clarify whether neurotoxicity associated with fragment B is real (i.e., due to the fragment itself) or only apparent (i.e., due to trace contamination with tetanus toxin).

Although fragment C did not possess toxicity, it did antagonize the neuromuscular blocking properties of tetanus toxin. This represents the first report that an atoxic fragment of a clostridial neurotoxin is capable of antagonizing the actions of a native toxin.

The ability of fragment C to antagonize the parent molecule was specific, as judged by two lines of evidence. Firstly, fragment C antagonized only tetanus toxin; it did not antagonize other presynaptic toxins such as botulinum toxin type A or β-bungarotoxin. Secondly, binding fragments that antagonize other toxins, such as cholera toxin (e.g., B subunit) and diphtheria toxin (e.g., CRM_{197}), did not

antagonize tetanus toxin, nor did they alter the effects of fragment C.

The interaction between fragment C and tetanus toxin appeared to involve the cell surface receptor. This conclusion is supported by the finding that fragment C antagonized unbound toxin, but it did not antagonize bound toxin. The latter result indicates that, under the conditions used in the present study, fragment C did not displace bound toxin.

Experiments with tissue homogenates confirmed the observatio that tetanus toxin was tightly bound. Homogenates of cerebral cortex adsorbed toxin from solution, and these homogenates competed with phrenic nerves for toxin in solution. However, homogenates of cortex did not promote desorption of toxin already bound to the phrenic nerve.

Relative to cerebral cortical tissue, eel and torpedo electric tissue did not adsorb much toxin. The failure of electric tissue to bind substantial amounts of tetanus toxin could mean the tissue has relatively few toxin receptors. Several groups have proposed that gangliosides are receptors for tetanus toxin (van Heyningen, 1974; Helting et al., 1977; Bizzini, 1978; Morris et al., 1980; Goldberg et al., 1981; Rogers and Snyder, 1981; but see Yavin et al., 1981). Interestingly, there are no reports on the ganglioside composition of synaptic membranes from electric organs. It would be worthwile to assay these tissues for gangliosides, and then to relate ganglioside content to toxin adsorbing ability.

As discussed thus far, the data suggest that fragment

C binds to phrenic nerves, competes with tetanus toxin for its receptors, but does not compete with other toxins for their receptors. The data do not, however, answer one especially important question: What is the nature of the receptor for which fragment C and tetanus toxin compete? There are no published data that permit one to decide whether the tetanus toxin receptor in brain is the same as the tetanus toxin receptor in peripheral nerve. In addition, there is no basis on which to decide whether the peripheral receptor mediating toxin-induced spastic paralysis is the same as that mediating toxin-induced flaccid paralysis.

The identity or non-identity of putative receptors can be determined by comparing their respective pharmacological properties. An ideal approach would be to compare the ability of a series of drugs to inhibit toxin binding at one receptor site with the ability of these same drugs to inhibit binding at another receptor site. Unfortunately, no drugs have been found that have high affinity for any tetanus toxin receptor, so this approach for comparing receptors cannot as yet be pursued.

An alternative is to determine the apparent K_D describing the binding of tetanus toxin or fragment C to central and peripheral receptors, and then to compare these kinetic constants. Goldberg et al. (1981) and Rogers and Snyder (1981) have reported that tetanus toxin and fragment C bind to brain tissue with a K_D in the nanomolar range. This is much lower than the apparent K_D obtained in the present

study, which was 1.4×10^{-7} M. However, the methods used were substantially different. Goldberg et al. (1981) did direct binding assays on non-functioning cells under conditions in which ligand binding was dissociable. The present study did indirect assays on functioning cells under conditions in which ligand binding was not dissociable. The marked difference in K_D values is probably a reflection of the difference in techniques used to generate the values.

Efforts are underway to study the binding of tetanus toxin to central and peripheral tissues under comparable conditions. However, it must be acknowledged that this will be difficult. There are relatively few problems associated with the experimental use of functioning peripheral nerves, but there are numerous problems associated with the use of functioning brain cells (see Collingridge and Davies, 1982). Conversely, many techniques have been described for isolating synaptic membranes from central nerves, but no techniques have been described for isolating workable quantities of synaptic membranes from phrenic nerves. These matters make it clear that studying the central and peripheral actions of tetanus toxin under strictly comparable conditions will not be easy.

C. The binding fragment of tetanus toxin versus botulinum toxin.

The fact that botulinum toxin and tetanus toxin are similar in their respective structures and pharmacological activities suggests that experiments should be done to deter-

mine whether they share a common receptor, a common mechanism for internalization, or a common intracellular substrate. The present report examines the first of these three possibilities. Data are presented which suggest that certain botulinum toxins and tetanus toxin recognize similar membrane determinants.

Several groups have shown that clostridial neurotoxins bind with high affinity to receptors in brain. The heavy chain of botulinum toxin competes with its parent molecule for binding sites (Kozaki, 1979; Williams et al., 1983), and the heavy chain of tetanus toxin competes with its parent molecule for binding sites (Morris et al., 1980; Goldberg et al., 1981). In contrast to work on the central nervous system, work on the neuromuscular junction has not demonstrated directly that there are high affinity binding sites for botulinum toxin or tetanus toxin. In fact, high affinity binding sites have not been demonstrated directly for any toxin that acts presynaptically at the neuromuscular junction (e.g., β-bungarotoxin, taipoxin, notexin, etc.) The relatively small number of nerve cells, combined with the presumed small number of binding sites, has posed serious methodological problems. However, work described in the previous section has provided indirect evidence for specific binding sites for tetanus toxin. It was shown that the binding fragment from tetanus toxin did not block neuromuscular transmission, but the fragment did antagonize the ability of native tetanus toxin to block neuromuscular transmission.

The techniques that were used to study tetanus toxin have now been used to study botulinum toxin. The data indicate that at the same concentration at which the binding fragment antagonizes tetanus toxin, the fragment also antagonizes neurotoxin types C and E.

In a series of preliminary experiments, the binding fragment of tetanus toxin was examined for its ability to antagonize each of the seven botulinum neurotoxins. The fragment did not produce detectable antagonism of five of the neurotoxins (types A, B, D, F and G). In relation to this apparent absence of antagonism, two points should be made. Firstly, the technique used to detect antagonism was, in essence, a bioassay (paralysis of hemidiaphragm), and this technique is not as sensitive as radioreceptor binding assays. This means there could have been antagonism of binding that fell below the limits of detection by bioassay. Secondly, the concentration of binding fragment that was tested was within the range of that which antagonizes tetanus toxin. Perhaps higher concentrations would have antagonized all botulinum neurotoxins, but these concentrations are above the range (> 10^{-6} M) of authentic pharmacological interest.

The interaction between the binding fragment of tetanus toxin and botulinum toxin type C was studied in some detail, and the results support two major conclusions: i.) the pharmacological actions of toxins and binding fragment were due to homogeneous preparations of protein that were free of significant contamination, and ii.) the antagonism of toxins

produced by binding fragment occurred at the level of the cell membrane.

Most pharmacological experiments with clostridial neurotoxins are not done with purified material. More typically, experiments are done with crystalline type A toxin, which is an aggregate of neurotoxin and hemagglutinin, or with impure preparations obtained from culture supernatants. In the present study, impure preparations were used to screen toxins that would interact with binding fragment, but a pure preparation of botulinum neurotoxin was used for subsequent experiments. Thus, type C botulinum toxin, as well as tetanus toxin and binding fragment from tetanus toxin, were all reasonably homogeneous, as judged by HPLC.

Evidence was also obtained to show that the binding fragment rather than any contaminant was responsible for antagonism of neurotoxins. Both the fragment and pharmacological activity eluted from G-25 columns at the void volume, indicating that salts and small molecular weight compounds were not involved. The fragment and pharmacological activity were retained during dialysis (molecular weight cutoff = 25,000), indicating that compounds of intermediate molecular weight were not involved. And finally, HPLC studies revealed that the only large molecule present in the binding fragment solution was the fragment itself.

The data indicate that binding fragment and botulinum neurotoxin interact at the level of the cell membrane. For example, tissues were incubated with proteins under conditions

(low temperature; no nerve stimulation) that allowed binding to go to completion but which retard internalization (cf. Simpson, 1980; Schmitt, 1981). Under these conditions, the binding fragment antagonized type C neurotoxin if it was added before the toxin but not if it was added after toxin. When viewed in the context that binding fragment did not inactivate the toxin molecule, the data strongly indicate competition for a membrane binding site.

Although the data suggest interaction at the level of the cell membrane, they do not necessarily imply interaction at the toxin receptor(s). Three possible explanations must be considered: i.) the receptor for tetanus toxin is the same as that for type C and/or type E botulinum toxin, ii.) the receptor for tetanus toxin is similar to but not identical with those for botulinum toxins, or iii.) tetanus toxin and botulinum toxin interact with a common molecule other than the receptor, such as a molecule that promotes internalization.

There is as yet no basis for deciding which of these possibilities is more likely. However, there is a line of investigation that may yield helpful answers. Van Heyningen and his associates have shown that tetanus toxin binds to gangliosides (Van Heyningen, 1959; Van Heyningen and Miller, 1961), and the finding has been reproduced (Morris et al., 1980; Rogers and Synder, 1981). Similarly, Simpson and Rapport (1971) showed that botulinum toxin binds to gangliosides, and their findings have also been confirmed (Kitamura et al., 1980). In all cases, toxin binding to gangliosides,

which are naturally occurring constituents of nerve membranes, inactivated the toxins. It has been proposed that gangliosides or sialoglycoproteins could be toxin receptors, or they could be molecules that mediate internalization. Conceivable, a determination of the role played by sialic acid containing molecules would help resolve the mechanism by which the binding fragment of tetanus toxin antagonizes botulinum toxin.

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